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Abstract

Excessive erythrocytosis results in severely increased blood viscosity that may compromise the vascular endothelium. Using our transgenic mouse model of excessive erythrocytosis we previously showed that despite altered brain endothelial cell morphology and an activated vasculature, brain vascular integrity was largely maintained up to 4-5 months of age. We now present data showing that persistent long-term damage of the vascular wall during the later stages of adulthood (9-10 months) results in a chronic detrimental inflammatory phenotype and increased vessel permeability that likely contributes to the reduced life span of our erythropoietin overexpressing transgenic mouse. In aged transgenic animals inflammatory cells were detected in brain tissue and elevated RNA and protein levels of inflammatory markers such as IL-6 and TNFα were observed in both brain tissue and blood plasma. Additionally, increased expression of p53 and other pro-apoptotic markers, as well as decreased Bcl-xL expression in the brain vasculature, indicated ongoing cell death within the vascular compartment. Finally, abnormally elevated vascular permeability in all organs was detected in correlation with decreased expression of the tight junction protein occludin and the adherens junction protein β-catenin in brain. Thus chronic erythrocytosis results in sustained activation of inflammatory pathways, vascular dysfunction and blood-brain barrier disruption.

Key words; erythropoietin, endothelium, cell death, endothelial activation

1. Introduction

Adequate oxygen delivery is crucially important for brain function because of its low capacity for anaerobic metabolism. The hematopoietic growth factor erythropoietin (Epo) circulates in plasma and controls the oxygen carrying capacity of the blood (Fisher, 2003; Velly et al.). Epo is produced primarily in the adult kidney and fetal liver and was originally believed to play a role restricted to stimulation of early erythroid precursors and differentiation of the erythroid lineage. In addition to its well known role in erythropoiesis however a diverse array of cells have now been identified that produce Epo and/or express the Epo receptor including endothelial cells, smooth muscle cells and cells of the central nervous system (Digicaylioglu et al., 1995; Masuda et al., 1994). *In vitro* Epo has been shown to regulate a variety of neural functions such as calcium flux (Korbel et al., 2004) neurotransmitter synthesis and cell survival (Velly et al., 2010; Vogel et al., 2003). Furthermore Epo has neurotrophic effects (Chen et al., 2007; Grimm et al., 2002; Junk et al., 2002), can induce an angiogenic phenotype in cultured endothelial cells, is a potent angiogenic factor *in vivo* (Ribatti et al., 2003) and enhances ventilation in hypoxic conditions (Soliz et al., 2005).

Clinically, due to its role in erythrocytosis, lack of Epo leads to anemia with fatigue and cellular hypoxia. Thus recombinant human Epo (rhEpo) is a frequently used therapeutic to increase red blood cell number and improve oxygen delivery. Excessive erythrocytosis however results in abnormally high blood viscosity and is often associated with severe clinical complications such as hypertension and thromboembolism (Bertinieri et al., 1998; Ruschitzka et al., 2000). Notably the effects of elevated Epo levels, and thus resultant high hematocrit, on blood vessel function and structure *per se* has not been fully

investigated. The integrity of blood vessel wall structure is essential to facilitate efficient blood transport/perfusion and the efficient oxygenation of all tissue beds and organs. Vascular remodelling to suit local tissue needs may therefore be an important adaptation to excessive erythrocytosis. Indeed endothelial cells that are in direct contact with high blood viscosity, may be altered and undergo changes in permeability and structure. Such alterations/modifications may compromise blood-brain barrier integrity and disturb neuronal homeostasis in the brain.

To study adaptive mechanisms to excessive erythrocytosis we have generated a mouse model (designated tg6) that constitutively over expresses human Epo in an oxygen-independent manner. Generation and characterization of this line showed these mice have a hematocrit of 0.8-0.9 after the first 8-9 weeks without increased blood pressure or altered cardiac output as well as reduced exercise performance and a significantly reduced life span of 9-12 months (Heinicke et al., 2006; Ogunshola et al., 2006; Ruschitzka et al., 2000; Vogel et al., 2003; Wagner et al., 2001). Interestingly both NO mediated relaxation and circulating NO levels are markedly elevated leading to increased vasodilation and protection from cardiac complications (Ruschitzka et al., 2000).

Using this transgenic model we previously investigated the direct effect of Epo and the impact of excessive erythrocytosis on angiogenesis, blood vessel wall structure and integrity in adult mice of 4-5 months of age (Ogunshola et al., 2006). We observed significant activation of the brain vascular endothelium however, surprisingly, blood-brain barrier permeability remained unaffected (Ogunshola et al., 2006). Thus compensatory mechanisms seemed to enable normal vascular function. This follow up work investigates the long term effects of chronic erythrocytosis in aged animals of 9-10 months. Our data shows that both inflammatory and cell death pathways are strongly

stimulated in the vascular compartment and likely cause wide-spread damage to the vascular endothelia. Accordingly, all organs display a significant increase in vascular permeability. Thus during the aging process sustained chronic excessive erythrocytosis activates multiple mechanisms that reduce organ function and ultimately contribute to premature death.

2. Results

2.1 Endothelial activation markers remain upregulated during aging in mice exhibiting excessive erythrocytosis

Previously we have shown that chronic excessive erythrocytosis results in activation of the brain vasculature in adult tg6 mice. We now investigated the activation status of the brain vasculature in aged tg6 mice of 9-10 months of age using immunohistochemistry for VCAM-1. Tg6 endothelium was found to be strongly VCAM-1 positive indicating continuous activation of the vascular bed (Fig. 1A) compared to wt animals. Inserts show high magnification of the vascular structures that stain positive for VCAM-1. Western blotting and quantification confirmed this result showing a very strong expression of VCAM-1 in brain lysates of tg6 but not wt animals. Notably, ICAM-1 protein levels were also upregulated (Fig. 1B).

2.2 Inflammatory mediators and cytokines are elevated in aged tg6 mice

To further ascertain the impact of chronic vascular activation we performed quantitative polymerase chain reaction (qPCR) analysis of mRNA from tg6 and wt brain tissue. We observed a 2-3 fold increase in both IL-1β and TNFα as well as modest but significantly elevated levels of VCAM-1 and iNOS (Fig. 2A). Thus expression of inflammatory cytokines is significantly increased in the transgenic animals. In blood plasma we also observed that circulating levels of IL-6 were more than doubled compared to wt mice (Fig. 2B), further indicating that inflammatory pathways are chronically induced in tg6 animals. We subsequently identified the main source of the systemically increased IL-6 levels as being the kidney, liver and spleen - organs that are known to undergo significant

degeneration during the aging of these mice (Fig. 2C, (Heinicke et al., 2006)). Although brain and heart tissue did not express significantly increased levels of IL-6 the systemically increased IL-6 suggested the possibility of increased infiltration of inflammatory cells within many tissues including the brain parenchyma. Accordingly, we detected a limited number of inflammatory cells usually absent from the normal brain in aged tg6 mice (figure 2D). Indicative of an impairment of the blood-brain barrier, the infiltrating macrophages ($CD11b^+$) and T-cells ($CD4^+$, data not shown) were frequently detected in or around blood vessels in the transgenic brain.

2.3 Increased cell death in tg6 brain vasculature

The inflammatory phenotype observed in aged tg6 mice suggested a significant detrimental effect on the vascular endothelial cells. Accordingly we observed reduced expression of Bcl-2 mRNA (Fig. 2A) and increased protein expression of pro-apoptotic proteins such as p53, Bax and Bnip3 in tg6 brain tissue compared to wt (Fig. 3A). Concomitantly, reduced levels of the anti-apoptotic protein Bcl-xL were also noted in tg6 brain lysates (Fig. 3B) further highlighting a significant increase in cell death pathways in these animals. Immunohistochemistry demonstrated that in the brain p53 is indeed mainly localized to CD31+ve blood vessels (Fig. 3C and D) and is elevated in tg6 mice underlining the reduced survival of cells within the vascular bed. Interestingly plasma levels of VEGF, a growth factor known to be very important for survival of many cells in the brain and other organs, was also observed to be significantly down-regulated (Fig. 3E).

2.4 Vascular integrity is compromised in aged tg6 mice

To observe alterations in vascular integrity in aging animals we performed Evans Blue extravasation assays on aged wt and tg6 mice. Unlike the results from younger animals in our previous studies (Ogunshola et al., 2006), all aged tg6 mouse organs had significantly increased leakage of Evans Blue into the tissue parenchyma compared to wt littermates (Fig. 4A). Notably, although the greatest flux was observed in the kidney, the normally highly impermeable brain vasculature also revealed significant leakage compared to wt animals. This data also correlated with Western blotting data from brain lysates showing a distinct decrease in expression of the tight junction protein occludin (Fig. 4B and C). The expression of β-catenin, a protein involved in maintenance of endothelial adherens junctions, was also significantly downregulated (Fig. 4B and C). Surprisingly, levels of claudin-5 and the adaptor protein ZO-1, other components of tight junctions, remained unaffected (Fig. 4B and C).

3. Discussion

Despite hematocrit values of 0.8-0.9 and an activated endothelium, blood-brain barrier permeability in 4-5 month old tg6 mice remained unchanged compared to wt animals (Ogunshola et al., 2006). Thus it appeared that the microvascular circulation is able to adapt and withstand the adverse effects of excessive erythrocytosis. However, we considered that sustained high levels of NO and chronic high blood viscosity observed in tg6 animals could eventually cause permanent damage to the vessel walls facilitating increased susceptibility to leakage over time. This current study supports this hypothesis and confirms that during the aging process sustained activation of inflammatory and stress response pathways culminate in elevated levels of inflammatory cytokines and compromised cell viability with pathological consequences. As a result permeability of all vasculature including the blood-brain barrier increased significantly in tg6 animals aged 9-12 months.

Vascular endothelial cells are constantly subjected to shear stress imposed on them by blood flow. Normal physiological shear stress is vasculoprotective and fosters quiescence of the endothelium and vascular wall. Indeed recent work suggests that shear stress may be the most critical factor affecting gene expression in endothelial cells and during inflammation. Data from *in vitro* (Stone et al., 2003; Walsh et al.; Warabi et al., 2004) and *in vivo* (Xu et al., 2004; Yamawaki et al., 2003) studies indicate that low or low oscillating endothelial shear stress upregulates molecular and vascular responses that may be instrumental to progression of vascular-related diseases. Excessive erythrocytosis compromises vascular homeostasis as a result of significantly increased hematocrit and decreased plasma levels (Neunteufl et al., 2001). We have previously shown that NO-mediated vasodilatation and increased blood viscosity are two ways by which tg6 mice adapt to the extremely high hematocrit levels (Vogel et al., 2003). However elevated blood viscosity significantly reduces blood velocity causing a concomitant lowering of shear stress to levels that are probably detrimental in the long term. On the other hand

significantly increased red blood cell content may indicate increased oxygen delivery. Interestingly, it was recently demonstrated that although blood viscosity is about four times higher in tg6, oxygen delivery is identical with wt mice again suggesting vascular compensatory mechanisms exist (Frietsch et al., 2007). The mechanisms underpinning this compensation are still largely unclear.

Progressive blood-brain barrier breakdown is associated with diverse CNS inflammatory conditions. Inflammation is thought to be important in the development and/or progression of vascular disease and is particularly associated with alterations in the expression of vascular and platelet adhesion molecules, levels of coagulation factors and activity of oxidants and anti-oxidants (Ross, 1999) such as observed in our mouse model. That excessive erythrocytosis results in a state of chronic and/or recurrent inflammation and endothelial dysfunction correlates with results from clinical studies of polycythemia vera patients who also exhibit endothelial dysfunction and perturbation that may predispose them to arterial disease (Falanga et al., 2000; Neunteufl et al., 2001). Together these observations suggest that endothelial damage is indeed associated with polycythemia vera.

Current evidence advocates that Epo protects many cell types against different injuries (reviewed in (Velly et al.; Vogel and Gassmann)). Regarding the vasculature Epo has been shown to increase endothelial cell survival during exposure to prolonged hypoxia *in vitro* (Chong et al., 2002) and prevent BLOOD-BRAIN BARRIER permeability by restoring junction protein localisation *in vivo* (Li et al., 2007; Martinez-Estrada et al., 2003). It has also been shown by other groups that Epo can directly interact with and reduce the number of leukocytes, and the inflammatory response as a whole, in both kidney failure patients and mouse model systems (Shurtz-Swirski et al., 2002). Thus current literature suggests that a negative effect of Epo on the vasculature is very unlikely although at present this possibility cannot be totally ruled out. We hypothesize however that the inflammatory state and increased cell death markers in tg6 endothelium are not

directly related to elevated Epo levels but rather due to the detrimental effect of chronic exposure of vessels to high blood viscosity and circulating immune cells. For example, high blood viscosity and subsequently reduced blood velocity may increase interactions of endothelial cells with leukocytes. Indeed tg6 mice have increased leukocyte plasma levels (Wiessner et al., 2001) and we also observed inflammatory leukocytes, including CD11b+ and CD4+ cells within the brain parenchyma. In healthy systemic conditions these cells are absent from the immune-privileged CNS emphasizing partial impairment of the blood-brain barrier in tg6 mice. The detection of IL-6 in blood plasma and increased expression of TNFα and IL-1β only underline the degree of ongoing inflammation within the brain and various tissues. The major source of the increased systemic IL-6 levels was identified to be the kidney, liver and spleen. These organs have already been shown to undergo significant degeneration in this mouse model and significantly contribute to reduced life span of the animal (Heinicke et al., 2006; Ogunshola et al., 2006; Ruschitzka et al., 2000; Vogel et al., 2003; Wagner et al., 2001). In addition a functionally disturbed endothelium was already detected in these organs in animals at 4-5 months and in the current study using aged mice increased permeability was observed in all organs including brain. Although we detected only moderate increases in pro-inflammatory cytokine levels, the long term effects of chronic endothelial activation combined with low-level production of pro-inflammatory cytokines likely culminates in reduced vascular integrity with detrimental consequences. Indeed inflammatory mediators such as IL-1β, TNFα and IL-6 have been shown to have a prominent role in blood-brain barrier disruption (Abbott, 2000; Argaw et al., 2006). Though the mechanisms by which they act remain largely unclear, it has been suggested that IL-1β affects paracellular permeability while TNFα increases transcellular permeability (Abbott, 2000). Furthermore, similar to activation molecules such as ICAM-

1 and VCAM-1, they can act directly or indirectly to attract leukocyte infiltration (Alvarez and Teale, 2006; Kevil et al., 2004). Such progressive inflammatory vascular disruption would therefore be associated with an endothelial pro-inflammatory phenotype, junctional complex remodeling and infiltration of leukocytes - all the parameters clearly noted in our study.

Epo-mediated protection against apoptosis was shown to upregulate Bcl-xL in cultured neurons (Wen et al., 2002), as well as increase Bcl-2 (Wei et al., 2006) and decrease Bax expression (Kumral et al., 2006) *in vivo*. In contrast we detected a significant increase in expression of pro-apoptotic proteins and a concomitant decrease in survival proteins in tg6 brain tissue and lysates. Clearly vascular endothelial cell apoptosis would exacerbate barrier dysfunction and accordingly increase vascular permeability as was observed in all organs. Indeed even small changes in brain integrity facilitate the influx of leukocytes and other circulating substances that can have enormous detrimental effects on neuronal function. Thus loss of vascular integrity in tg6 brain, although moderate, highlights that the normally highly impermeable blood-brain barrier has been compromised with likely severe consequences. We conclude that Epo-dependent and -independent protective mechanisms cannot be maintained ad infinitum during the aging process but are overwhelmed by the progressively activated endothelium and continuous inflammatory state. Thus the morphological brain endothelial changes we observed in younger animals (Ogunshola et al., 2006) represent early signs of a gradual but persistent chronic endothelial disturbance prior to complete breakdown with fatal consequences. The scheme in figure 5 indicates the observed mechanisms that may contribute to vascular compromise.

Overall this study shows that as a result of chronic excessive erythrocytosis significant vascular changes occur in the brain endothelium, and also in other organs during aging. After a period of months the cumulative effect of continued inflammation and pro-apoptotic signaling seem to dominate. As this mouse line has a significantly reduced life

span we conclude that these alterations substantially contribute to the premature death of the animal. We propose this animal model to study different stages of endothelial dysfunction and vascular changes that could play a central role in the development and progression of chronic mountain sickness, excessive erythrocytosis and other vascular-related diseases that cause/culminate in high hematocrit levels or are associated with endothelial activation.

4. Experimental procedures

All experiments were performed in accordance with Swiss animal protection laws and Zürich University institutional guidelines. The transgenic mouse line was generated by pronuclear injection of the full length human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter and has been previously described (Ruschitzka et al., 2000). The resulting mouse line TgN(PDGFBEPO)321ZbZ (termed tg6) showed increased plasma and brain levels of Epo (Wiessner et al., 2001) and was bred by mating hemizygous males to wild type (wt) C57BL/6 females. Half the offspring were hemizygous for the transgene and the other half were wt and served as controls. Brain tissue from male mice 9-12 months of age were used in all experiments. Of note, similar data was also obtained using female animals.

4.1 Evans blue permeability assay

Evans blue dye extravasation was used to assess vascular permeability. A 1% solution of Evans blue dye was injected intravenously into mice via the tail vein at a concentration of 2μg/g mouse weight. The dye was allowed to circulate for 4 hours before the animals were anaesthetised intraperitonally with ketamine/xylazine and then perfused

intracardially with ice cold PBS. After 15 minutes slow perfusion, the organs were dissected and the dye retained in the tissue parenchyma extracted with formamide at a concentration of 5µl/mg weight organ for 72 hours at room temperature. Absorbance was then measured at 620nm. Additional experiments were also performed allowing the Evans blue to circulate for only 45 minutes before anaethesis and perfusion. Results obtained were consistent using both methods and no significant differences were observed between male and female mice.

4.2 Quantitative polymerase chain reaction (qPCR)

RNA from mouse brain was extracted by homogenisation in TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using random hexamers and AMV reverse transcriptase (Promega). The cDNA equivalent to 50 ng of total RNA was PCR-amplified in an ABI PRISM 7700 detection system (PE-Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and quantified using the 2-ΔCT method using 18s rRNA as a housekeeping gene. Relative RNA levels are expressed as x-fold variations compared to control. Primers and probes for Taqman analysis for IL-1β, TNFα, and iNOS were purchased from Applied Biosystems. Primers and probes for Bcl-2 and VCAM-1 were purchased from Microsynth, Switzerland.

4.3 ELISA

Quantitation of VEGF and IL-6 were performed using ELISA kits according to manufacturers instructions (R&D Systems GmbH, Germany). Optical density was

measured using a microplate reader (Multiskan RC; Thermo Labsystems, Finnland) at 450nm with wavelength correction at 570nm.

4.4 Western blotting

Experiments were carried out on brain lysates from transgenic and littermate controls. Tissue was homogenised in lysis buffer by hand using a sterile dounce homogeniser. Samples were then centrifuged at 13,000 rpm at 4°C for 10 minutes, supernatant extracted and stored at –20°C. 50μg of protein were run on a 10% SDS PAGE, transferred to a nitrocellulose membrane, blocked in 5% non-fat milk and then incubated with primary antibodies overnight at 4°C or 2 hours at room temperature. Membranes were washed with either TBS or PBS containing 0.05% tween and then incubated for 1 hour with a horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection was carried out and the membrane exposed to Kodak autoradiography film. Normalisation was performed by reprobing filters with β-actin (1:5000, Sigma, Saint Louis, USA).

4.5 Antibodies

The following antibodies were used in this study; anti-occludin, anti-ZO-1, anti-Claudin-5 (1:1000, Zymed, San Francisco, USA), anti-β-catenin, anti-Bcl-xL (both 1:1000, BD transduction Labs), anti-ICAM-1, anti-VCAM-1 (intercellular and vascular cell adhesion molecule-1; both 1:50, Santa Cruz Biotechnology, Santa Cruz, USA) p53, Bax (1:50, Millipore), Bnip3 (1:500, Abcam).

4.6 Immunohistochemistry

Mouse brains were dissected and immediately frozen in liquid nitrogen. 10μM frozen sections were cut using a cryostat. Sections were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5min at room temperature then permeabilised in 0.1%Triton X-100 for 2min and blocked with 4% normal goat serum. Sections were then incubated with primary antibodies anti-p53 (1:1000, Santa Cruz, CA), anti-VCAM-1 (C8,1:500, Santa Cruz, CA) or anti-CD11b overnight at 4°C. Secondary antibodies (AlexaFluor 488 and CY5; Molecular Probes, Leiden, Netherlands) were then applied for 2 hours at room temperature followed by counterstaining with DAPI.

4.7 Statistical analysis

Graphic and statistical analyses were performed using Microsoft excel and Prism software. Western blot data was quantified using NIH Image J software. Data are presented as mean ± standard deviation (S.D). Statistical significance ($P< 0.05\%$) of the obtained data was calculated using two-tailed unpaired t-test.

5. Conflict of Interest

The authors declare that they have no conflict of interest.

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7. Figure legends

Figure 1: Persistent endothelial cell activation in aged tg mice

A) Fluorescent microscopy shows expression of VCAM-1 is localized to vascular structures within cortical brain sections and significantly increased in tg6 compared to wt control mice. Scale bar = 100μM. B) Western blotting and quantification of tissue homogenates from tg6 and wt brain show VCAM-1 and ICAM-1 are significantly upregulated in the transgenic mice. β-actin was used as a loading control. All samples for ICAM-1 were run on the same blot however the image was cropped due to redundancy as indicated.

Figure 2: Inflammatory pathways are strongly induced in tg6 mouse brain

A) Quantitative PCR of various inflammatory cytokines, iNOS, VCAM-1 and Bcl-2 using mRNA isolated from tg6 and wt brain. Bars represent mean ± SEM. *P<0.05, **P<0.01 versus wt control; n=4. B) IL-6 levels in blood plasma were measured by ELISA. A significant increase in systemic IL-6 levels was detected in tg6 plasma compared to wt littermates (**=p<0.005; n=5). C) Levels of IL-6 are significantly elevated in major organs namely kidney, liver and spleen but not heart or brain. D) Immunohistochemistry analysis revealed the presence of CD11b-+ve cells in the brain parenchyma of tg6 mice. No infiltrating cells were detected in wt mice (data not shown). Scale bar = 50μM.

Figure 3: Cell death pathways are activated in tg6 brain

Western blotting and quantification of brain protein lysates reveal that in tg6 animals A) expression of pro-apoptotic proteins are strongly induced compared to wt controls. B) In contrast, expression of the anti-apoptotic protein Bcl-xL is reduced in tg6. β-actin was used as a loading control. For each protein all samples were run on the same blot however the indicated images were cropped due to redundancy. C) Immunohistochemistry suggests that p53 (green) is localized mainly to vascular structures within the brain. Scale bar = 100μM. D) Confirmation of p53 expression by vascular cells by performing double staining with CD31. Scale bar = 50μM. Note increased intensity of stain in tg6 vessels. E)

Expression of VEGF, an important cytoprotective growth factor, was also down regulated in tg6 brain. (**=p<0.005; n=4).

Figure 4: Vascular permeability is increased in aged tg animals

A) Extravasation of Evans blue dye into tissue parenchyma was measured in organs of tg6 and wt mice. Significantly increased permeability was observed in all tg6 organs compared to control animals (*=p>0.05, **=p<0.005; n=12). B) Protein levels of the tight junction protein occludin are down regulated in tg6 brain lysates but in contrast claudin-5 levels remain unaffected. Tg6 mice also revealed significantly decreased β-catenin expression whereas ZO-1 and P-gp were unaffected compared to wt littermates. β-actin was used as a loading control. C) Quantification of all Western blots is shown. For each protein, all samples were run on the same blot however indicated images were cropped due to redundancy.

Figure 5: Schematic illustration of parameters involved in increased vascular permeability and blood-brain barrier breakdown in tg6 mice. We suggest that prolonged excessive erythrocytosis leads to increased expression of inflammatory mediators and sustained endothelial activation. This results in decreased expression of enothelial tight and adherens junction proteins as well as increased cell death culminating in elevated vascular permeability in all organs including brain.

Figure 1

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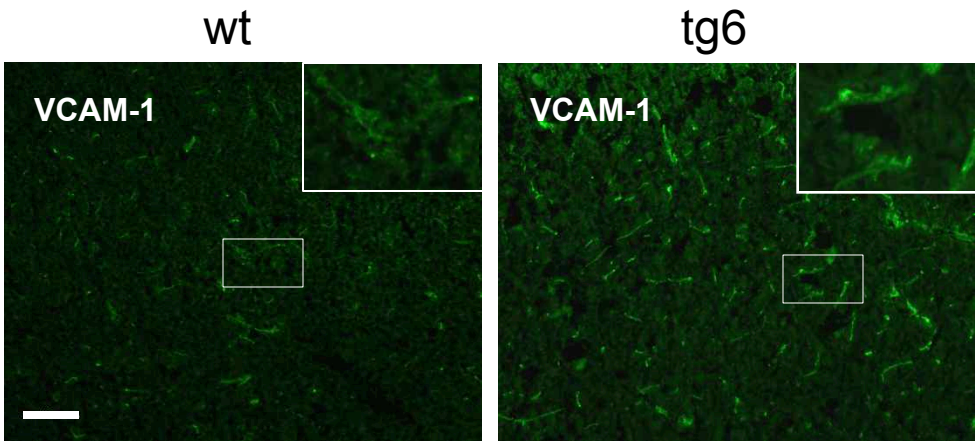
a



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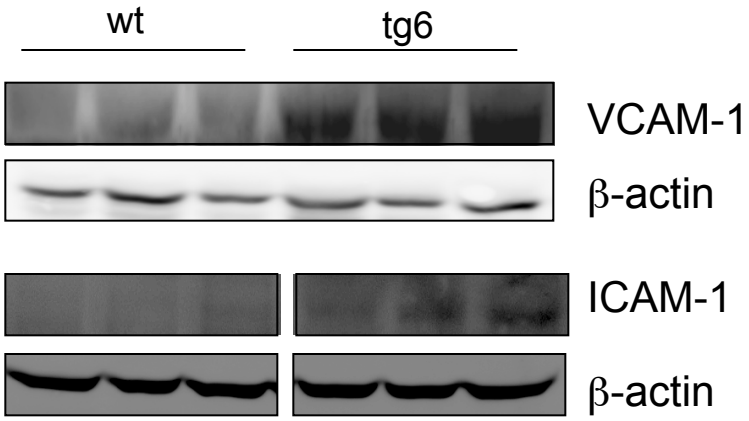

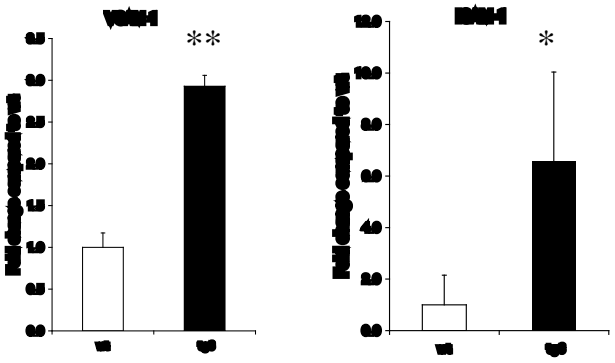

Figure 2

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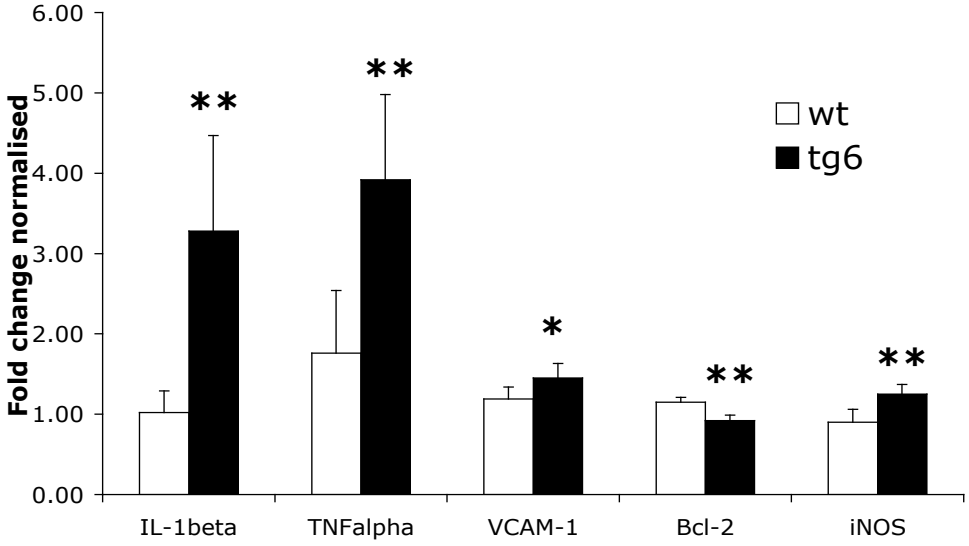
a



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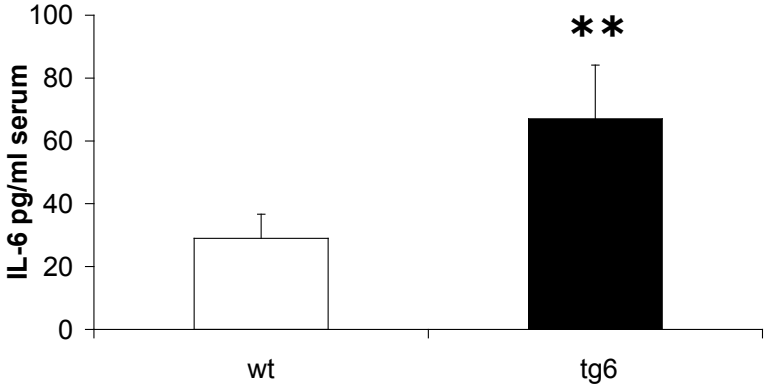

Figure 2

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c

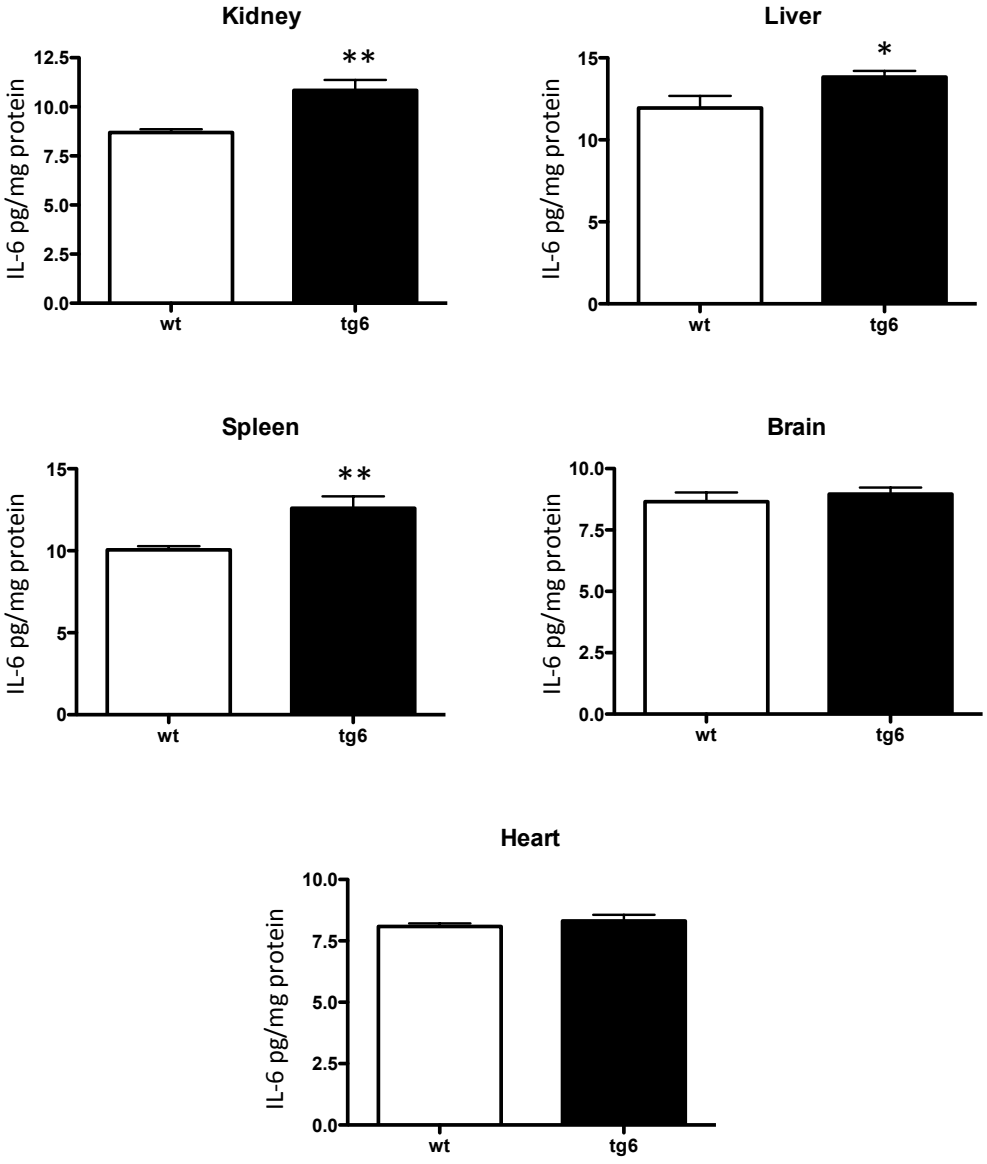

Figure 2

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d

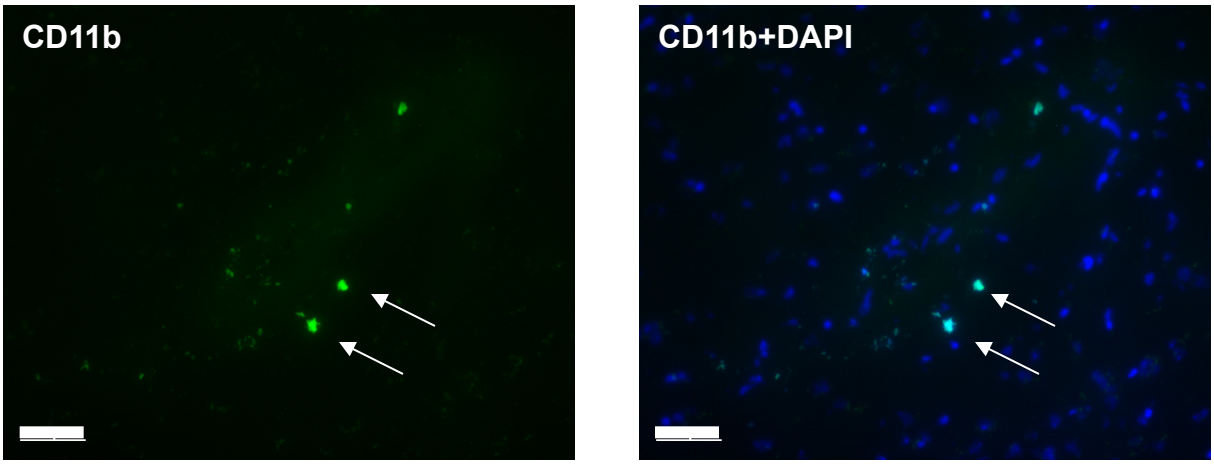

Figure 3

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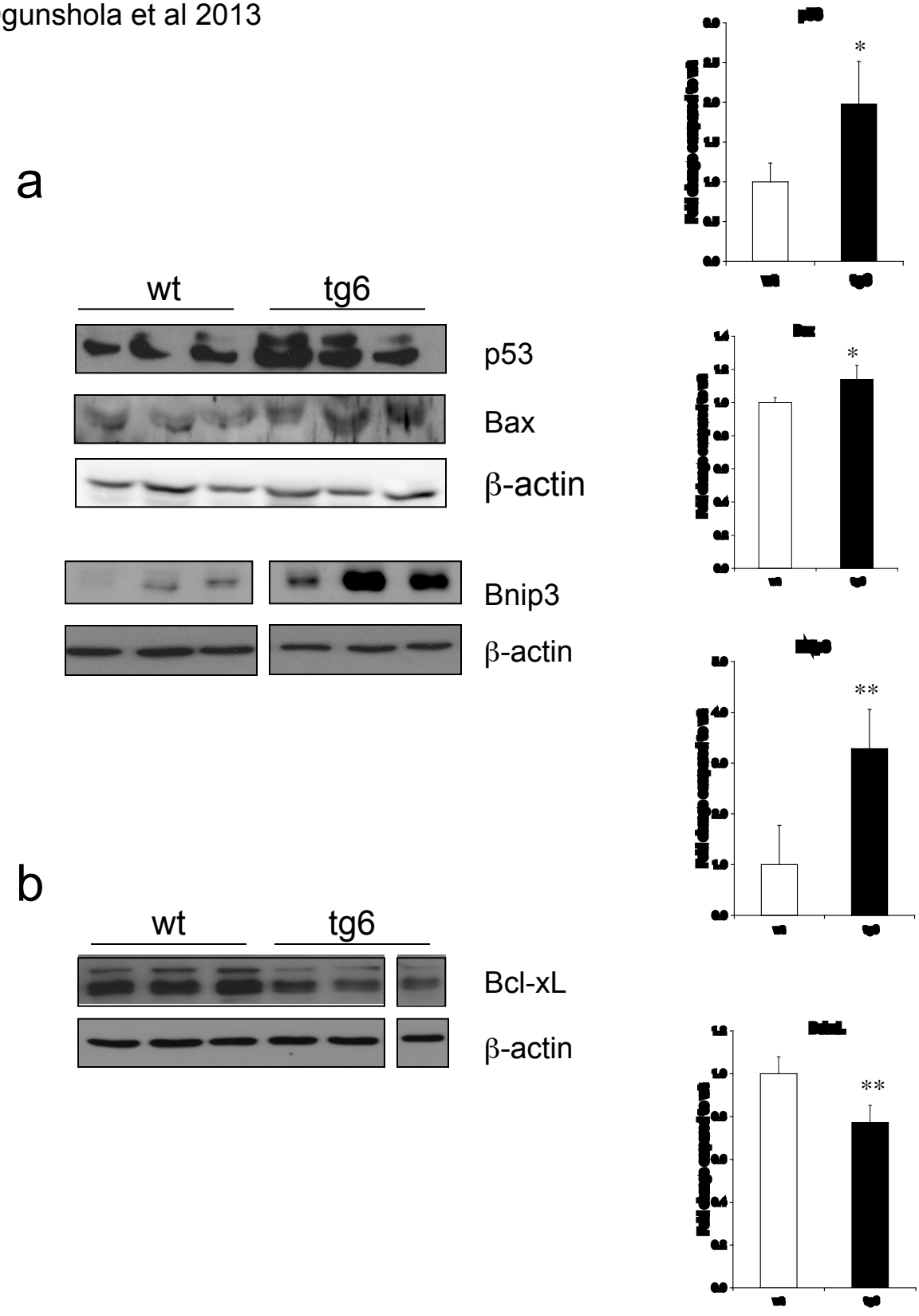

Figure 3

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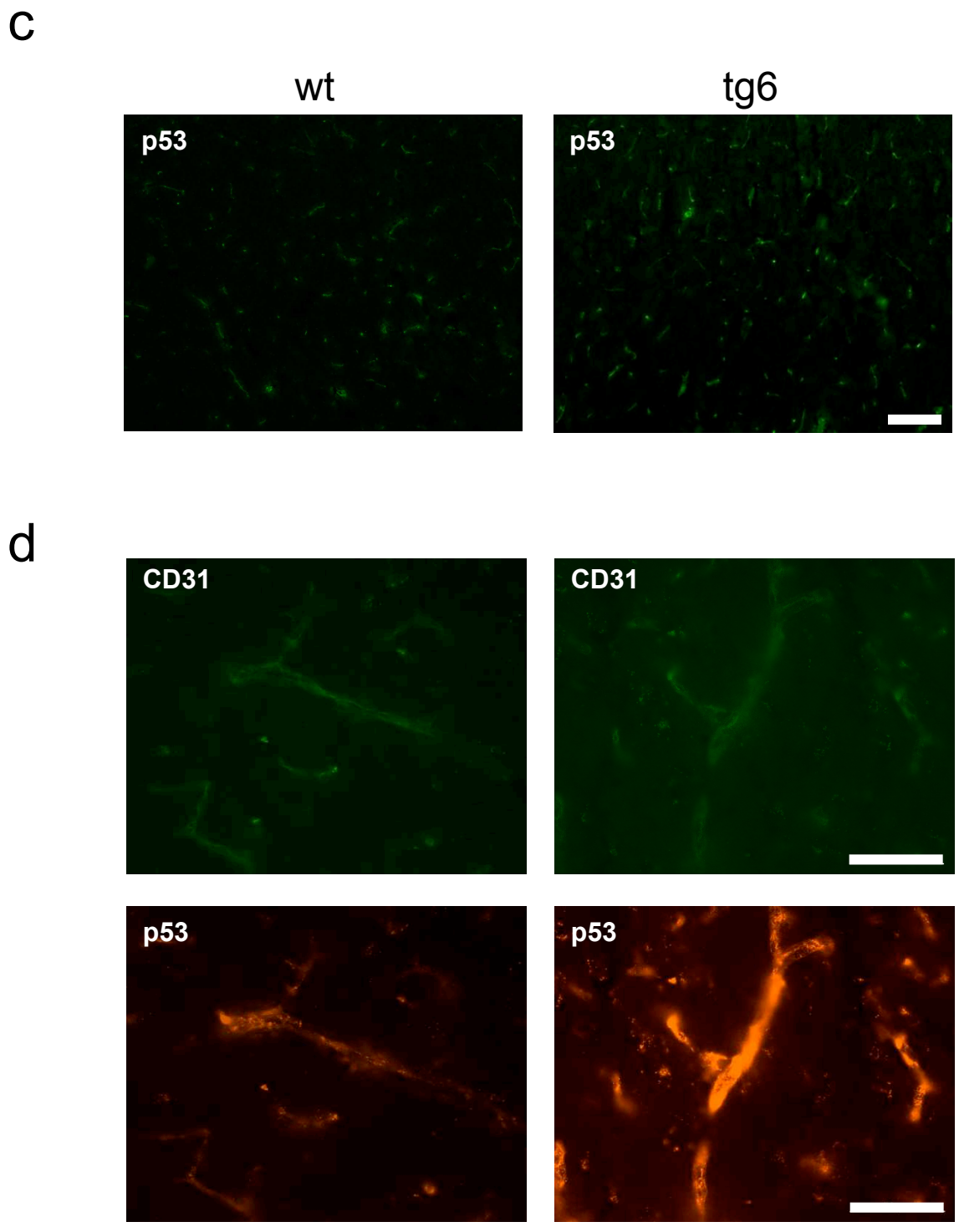

Figure 3

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e

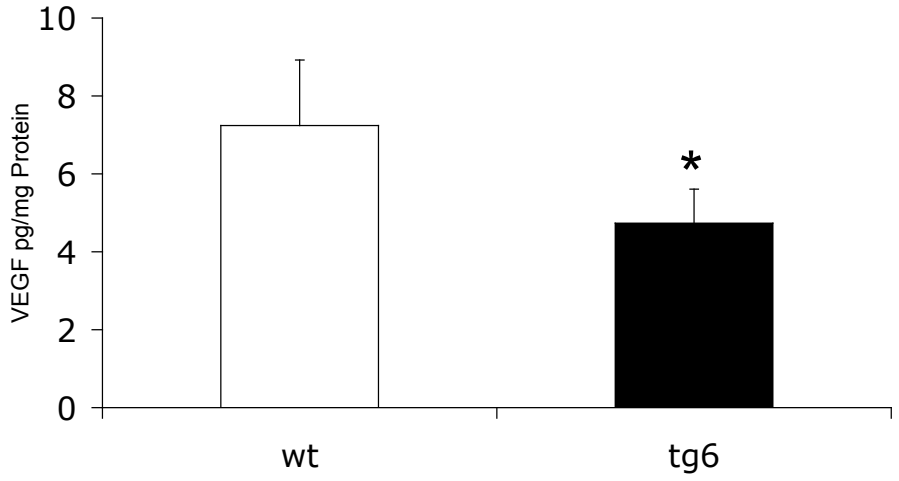

Figure 4

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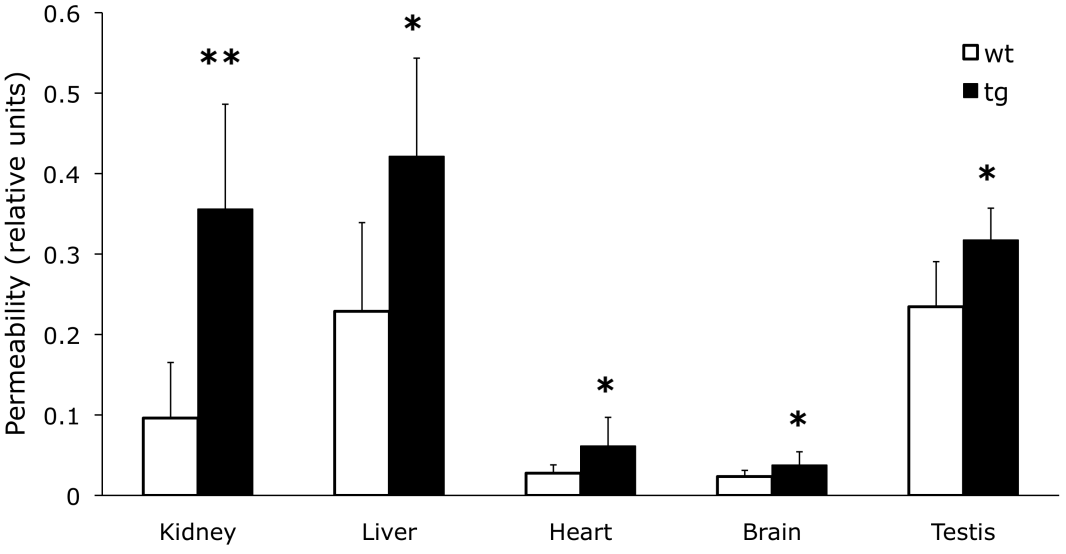
a



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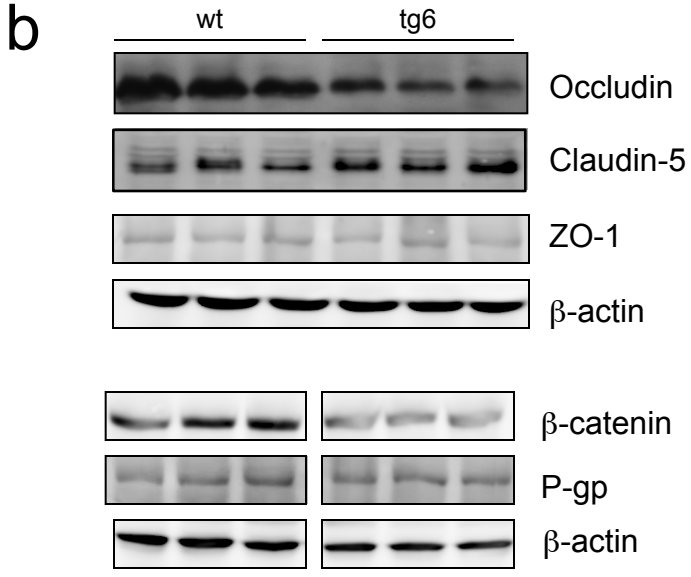

Figure 4

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c

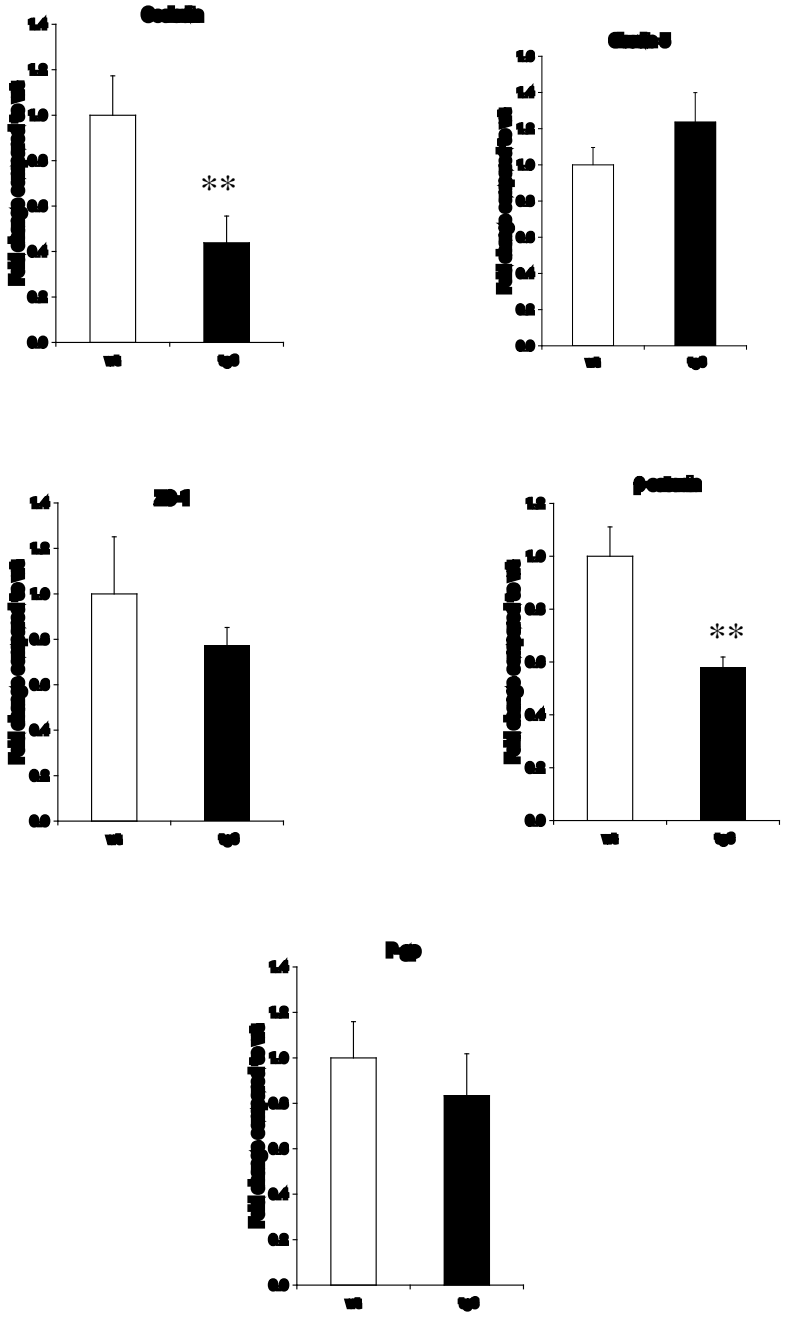

Figure 5

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